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COMPARATIVE STUDY ON MEMBRANE SOLUBILISATION OF BIOSYNTHESIZED NANO-SILVER & BIOSYNTHESIZED NANO-ZINC OXIDE ON SELECTED SPERM PARAMETER

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ABSTRACT

Extensive applications of nanoparticles are used in the field of catalysis, biosensing, imaging, drug delivery, nano-device fabrication and medicine. Previously, biosynthesized nano-silver and biosynthesized nano-zinc oxide has shown significant effect as antimicrobial agents, thus reduces concern over the threat of antibiotic resistance. Biosynthesis of nano-silver and nano-zinc oxide using *Pandanus amaryllifolius* leaf extract and their spermicidal effect was explored in the present research following characterization using FESEM-EDX and were determined to be spherical in shape and aggregated into regular structure with high uniformity. The sperm membrane solubilisation property displayed by both biosynthesized nano-silver and biosynthesized nano-zinc oxide exceeded the effects of 1% (w/v) Triton-X and was most significant at the concentration of 100 µg/ml. The results suggested that biosynthesized nano-silver and biosynthesized nano-zinc oxide holds possibility as new generation spermicidal agents.

Key words: Sperm membrane, cytotoxicity, nanoparticles, green synthesis, FESEM

INTRODUCTION

Nanotechnology has been used in many applications in daily life in the forms of nanomaterials and or nanoparticles (Gromadzka Ostrowska *et al.*, 2012) and can be synthesized using, bacteria, fungus and plant extract as the reducing agents (Logeswari *et al.*, 2012). Examples of widely used nanomaterials include copper, zinc, titanium, magnesium, gold and silver (Roy *et al.*, 2013). However, silver nanoparticles have had an extensive application in nanotechnology field (Logeswari *et al.*, 2012) and are used in materials science, antibacterial activity, catalytic properties biosensors, superconductors and medicine (Braydich-Stolle *et al.*, 2010). In medicine field, silver nanoparticles are incorporated as elements in implant surfaces, catheters, dental alloys, drug deliveries in cancer and renal therapies (GromadzkaOstrowska *et al.*, 2012). Despite its extensive application, silver nanoparticles exhibit cytotoxicity (Roy *et al.*, 2013). This cytotoxicity property can be useful in combating diseases such as cancer (GromadzkaOstrowska *et al.*, 2012). However, study on its potential as a spermicidal agent is still lacking. Hence, our study will focus on the potential of nanoparticles as a spermicidal agent and possibly be developed as a form of contraceptive. Certain medical conditions call the use of contraceptives by patients. The criteria set forth and defined by WHO as medical eligibility criteria enlists factors such as predisposition to DVT, pelvic inflammatory disease, vaginal bleeding, history of drug use and sexually transmitted diseases (STDs) and the dreaded HIV/AIDS infection (Barnard and Aston, 2012). The use of contraceptives in this case is a form of curbing unwanted pregnancies, preventing pregnancy-related health risks in women, reducing infant mortality and reducing if not preventing the spread of sexually transmitted diseases (STDs) as well HIV/AIDS (Enginsu *et al.*, 1991). Currently available contraceptives mainly focus on devices and methods for controlling pregnancies in women. However, methods such as hormonal contraceptive are strenuous on the body and may elicit long term effect. Therefore, an alternative method or device for male users is a welcoming contribution the industry. Options for

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male contraceptives are spermicide, condoms, abstinence, outercourse, vasectomy and withdrawal. The most preferred option is the spermicide (Chhonkera *et al.*, 2014; Tryphonas and Buttar, 1986). Most common ingredient used in spermicide is nonoxynol-9, a non-ionic surfactant mixture (Chhonkera *et al.*, 2014). Silver nanoparticles exhibit properties as a potential alternative ingredient in spermicides. One study clarified that the smaller-sized silver nanoparticles (10 - 25 nm) decreased the growth of male stem cells when they were exposed at concentrations greater than 10 µg/ml (Braydich-Stolle *et al.*, 2010). This is because of a greater surface to volume ratio achieved with nanoparticles, causing it to become very reactive (Braydich-Stolle *et al.*, 2010). The reactivity of the particles will promote apoptosis or the production of reactive oxygen species on the sperm cell (Braydich-Stolle *et al.*, 2010). Hence, the toxicity of silver nanoparticles can be used as spermicide agent.

MATERIALS AND METHODS

Animals for study

Sexually matured male rats (7-8 weeks old) were used in the experiment. Rats were fed with standard commercial rat pellets (Gold Coin Feed Mills (M) Sdn. Bhd) and water given *ad libitum*. Rats were maintained under standard conditions of humidity, temperature with 12 hrs of light/12 hrs of dark cycle. The test rats were allowed to acclimatize for one week prior to the experiment. All experimental procedures and animal maintenance were conducted in accordance to ethical approval by the university's UiTM CARE ethical committee The testes together with the epidymides were exposed via dissection before being excised, macerated to release sperm and incubated in PBS solution at 37°C (GromadzkaOstrowska *et al.*, 2012).

Biosynthesis of Nano-Silver and Nano-Zinc oxide particles

An alternative reducing agent of plant origin has been used to reduce the metals in the respective silver nitrate and zinc nitrate solution in the biosynthesis of nano-silver particles and nano-zinc oxide particles. *Pandanus amaryllifolius* aqueous extract was prepared by boiling the leaves in deionized water and filtering with Whatman No. 1 filter paper to remove the plant matter (Farooqui *et al.*, 2010). The aqueous solution was left to cool prior to use in the biosynthesis reaction. Biosynthesis of nano-silver particles was conducted by adding 5 mM silver nitrate ($AgNO_3$) solution to *Pandanus amaryllifolius* aqueous extract at a ratio of 2:1 and incubated at 37° C for 24 hrs on an orbital shaker. The solution turned from a light green solution to a dark brown solution indicating the presence of silver (Lanje *et al.*, 2010). To collect the silver particles, the solution was vortexed prior to being transferred into a falcon tube and centrifuged at 8000 rpm for 15 min. The centrifugation process was repeated twice to ensure maximum yield of the nano-silver particles. The supernatant was discarded and the nano-silver pellet was re-dispersed with distilled water. The suspended nano-silver solution was oven dried at 60° C for 24 hrs to ensure loose powder of nano-silver particles is obtained (Lanje *et al.*, 2010). The nano-silver particles were subjected to analysis under Field Emission Scanning Electron Microscope with Energy Dispersive X-ray (FESEM-EDX) for confirmation of particle size and presence of silver element. Biosynthesis of nano-zinc oxide particles was conducted by adding 5mM zinc nitrate ($Zn(NO_3)_2$) solution to *Pandanus amaryllifolius* aqueous extract at a ratio of 2:1 and incubated at 80°C at pH 8 (attained by adding NaOH). The resulting solution was subjected to constant stirring at 80°C for complete reaction to occur. The solution was allowed to cool to room temperature before collecting the clear supernatant to be dried in the oven at 60°C for 24 hrs. The resulting yellowish-white powder was subjected to analysis under Field Emission Scanning Electron Microscope with Energy Dispersive X-ray (FESEM-EDX) for confirmation of particle size and presence of zinc oxide element. Three concentrations of nano-silver and nano-zinc oxide were prepared to concentrations of 10 µg/ml, 50 µg/ml and 100 µg/ml respectively for use in subsequent analysis.

Incubation of sperm with biosynthesized nano-silver or nano-zinc oxide particles

Equal volumes of sperm suspension were incubated with 10 µg/ml, 50 µg/ml and 100 µg/ml concentrations of either nano-silver or nano-zinc oxide particles in microcentrifuge tubes. The solutions were left to incubate for 10 min prior to analysis on sperm membrane integrity (Olmsted *et al.*, 2000).

Sperm membrane integrity analysis

The control and treatment groups from the incubation with biosynthesized nanoparticles stage were analyzed for sperm membrane integrity for determination of functional sperm through the osmoregulatory capacity under hypo-osmotic conditions of 150 mOsm/L (Barnard and Aston, 2012). Preparation of swelling solution and procedure were conducted as outlined in (WHO, 2010). Briefly, 0.735 g of sodium citrate dihydrate and 1.351 g of D-fructose were dissolved in 100 ml of purified water. The sperm sample to hypo-osmotic

solution was mixed in a ratio 1:1 and incubated at 37°C for 30 min to evaluate membrane integrity. Following incubation period, sperm were stained with Diff-Quik solution (Moska *et al.*, 2011; Enginsu *et al.*, 1991) and scored as having intact membrane integrity by visual scoring of swollen tails based on WHO guidelines (WHO, 2010). A minimum of 100 sperm cells were evaluated per data entry.

Sperm motility determination

The control and treatment groups from the incubation with biosynthesized nanoparticles stage were analyzed for total motility. A volume of 10 µl for each tube was measured and pipetted onto the grid of a Makler chamber and observed under 400x magnification (WHO, 2010). A number of 100 sperm cells per data entry was observed and movement was recorded as progressive motility, non-progressive motility and immotile sperm.

Statistical analysis

Statistical analysis which was used is the ANOVA in order to analyze the significant difference between the mean of normal and abnormal morphology; and the significant difference between the mean of progressive motility, non-progressive motility and immotile sperm. Data was expressed as mean±SEM.

RESULTS AND DISCUSSION

Characterization of biosynthesized nano-silver and nano-zinc oxide particles

Biosynthesis of nanoparticles either nano-silver or nano-zinc oxide was evident from the initial and nearly immediate formation of brown precipitate in the mixture solution comprising *P. amaryllifolius* extract and silver nitrate solution or *P. amaryllifolius* extract and zinc nitrate solution (Suriyakalaa *et al.*, 2013). In this study *P. amaryllifolius* extract was used as a reducing agent. The extract was able to reduce the metal as the extract contains alkaloids, proteins, enzymes, amino acids, alcoholic compounds, and polysaccharides which are the compounds conferring the power of reduction of the silver ions to silver metal nanoparticles and zinc oxide ions to zinc oxide metal nanoparticles (Mittal *et al.*, 2013). Other compounds such as water soluble antioxidants, poly-phenol components, quinol and chlorophyll pigments which were also present in *P. amaryllifolius* extract was also the contributing factors for the reduction of ions and stabilization of nanoparticles (Tripathi *et al.*, 2013). Both types of nanoparticles were characterized using Field Emission Scanning Electron Microscope Energy Dispersive X-ray (FESEM-EDX). FESEM-EDX was employed to observe the morphology and elemental distributions at microscopic level (Shi *et al.*, 2011). Figure 1A is of nano-silver particles and Figure 1B is of nano-zinc oxide particles obtained using FESEM-EDX to show the dispersion, morphology of particles and determination of particle diameter. Results of FESEM-EDX have demonstrated that the nanoparticles has aggregated into nanoclumps as a result of stabilization process during biosynthesis (Mittal *et al.*, 2013). The range of diameters for the resulting nanoparticles was recorded between the values of 53-90 nm. It is postulated that the particle size is controlled by the concentration of plant extract used and the period of reaction time (Kora *et al.*, 2010). From our study, the concentration *P. amaryllifolius* extract and the duration of incubation was directly proportional to the reducing capabilities of the extract to reduce the ions to metals of nanosize (Kora *et al.*, 2010). Dispersion of the nanoparticles also shows a homogenous distribution with uniform spherical shaped particles. The spherical shaped particles have higher surface area as compared to other possible types. This leads for greater toxicity affect as the surface area per volume ratio of the nanoparticles (both nano-silver and nano-zinc oxide) which comes into contact with the sperm cell increases thus creates greater toxicity towards the sperm cell (Panyala *et al.*, 2008).

Sperm membrane solubilisation after incubation with biosynthesized nano-silver and nano-zinc oxide particles

Incubation of sperm with either nano-silver particles or nano-zinc oxide particles showed an increase in the occurrence of sperm with abnormal morphology as the concentration of biosynthesized nanoparticles was increased. Previous study had shown that as concentration of nanoparticles increased, the higher the surface area per volume ratio (Bahmanzadeh *et al.*, 2008). The higher surface area per volume will cause more sperm to be in contact with the nanoparticles (Braydich-Stolle *et al.*, 2010) conferring a higher degree of toxicity effect towards the sperm (Panyala *et al.*, 2008). As the toxicity of biosynthesized nano-silver and nano-zinc oxide particles towards the sperm increases it resulted in more sperm exhibiting abnormal morphology due to membrane solubilisation as a direct result of increase in osmotic pressure of the incubation solution. Abnormal sperm are not capable in fertilizing. Hence, reduces the fertility potential of an individual. Post hoc analysis showed that there is significant differences in normal and abnormal sperm morphology between each treatment of 10 µg/ml silver nanoparticles, 50 µg/

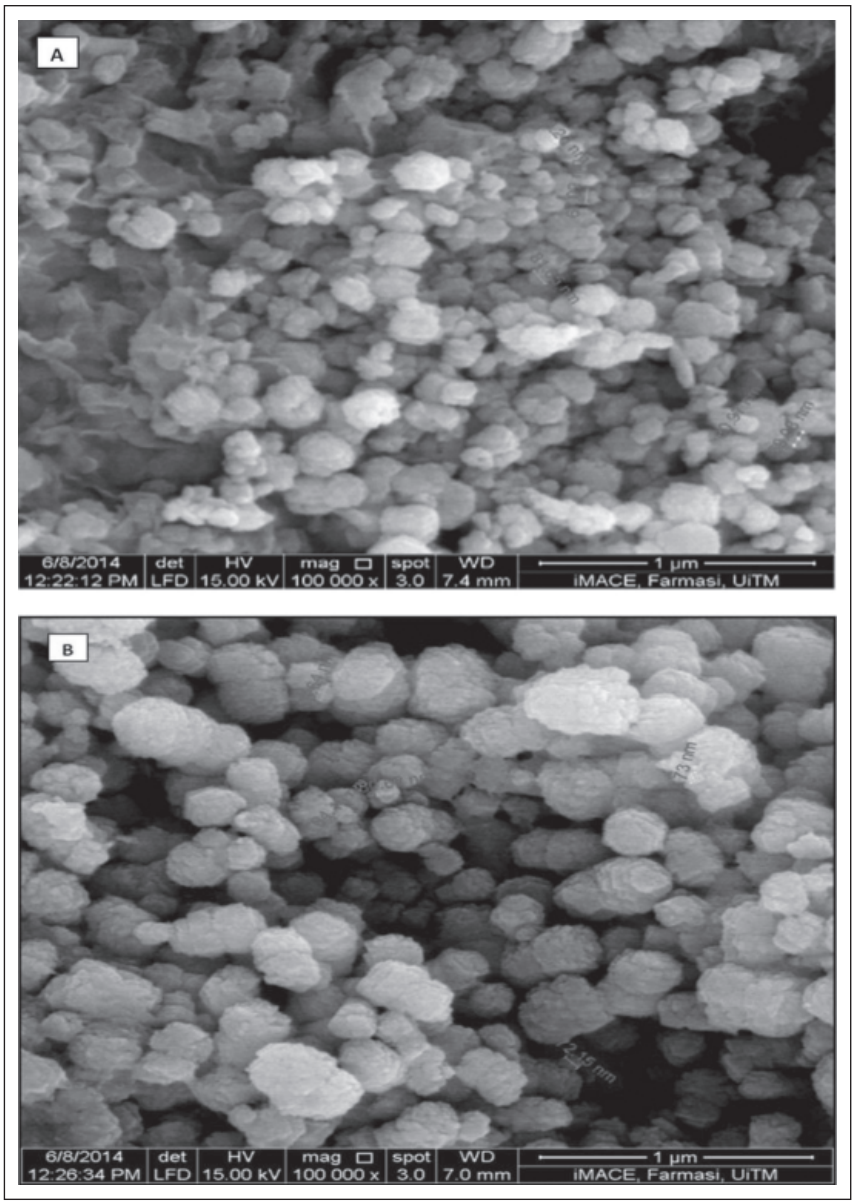

Fig. 1. FESEM-EDX analysis of particle dispersion, morphology and determination of particle diameter of nano-silver particles (Fig. 1A) and nano-zinc oxide particles (Fig. 1B) at 100,000x magnification.

ml silver nanoparticles and 100 µg/ml silver nanoparticles ($p<0.05$). Figure 2 shows the occurrence of abnormal sperm morphology for sperm incubated with biosynthesized nano-silver particles. Figure 3 shows the occurrence of abnormal sperm morphology for sperm incubated with biosynthesized nano-silver particles. Results from both experiments show that there is a stark increase in occurrence of abnormal sperm morphology. The observation is more evident for the 100 µg/ml nano-zinc oxide particles, where values for abnormal sperm morphology exceeded the value recorded for positive control group (Triton-X). Based on the study on the treatment of three different concentrations of biosynthesized nanoparticles, all treatment groups show increasing toxicity effect than negative control and/or positive control group. Post hoc analysis revealed there is significant differences in sperm morphology between negative control and all the treatment groups of biosynthesized nanoparticles ($p<0.05$). However, biosynthesized nano-silver particles were more effective at solubilising the membrane as compared to nano-zinc oxide. Values of sperm with abnormal morphology from nano-silver particles recorded higher percentages for observation. Evidence therefore suggests that both types of biosynthesized nanoparticles have effect on the sperm morphology even at low concentrations and thus has potential to be developed into spermicidal agents (Ema *et al.*,

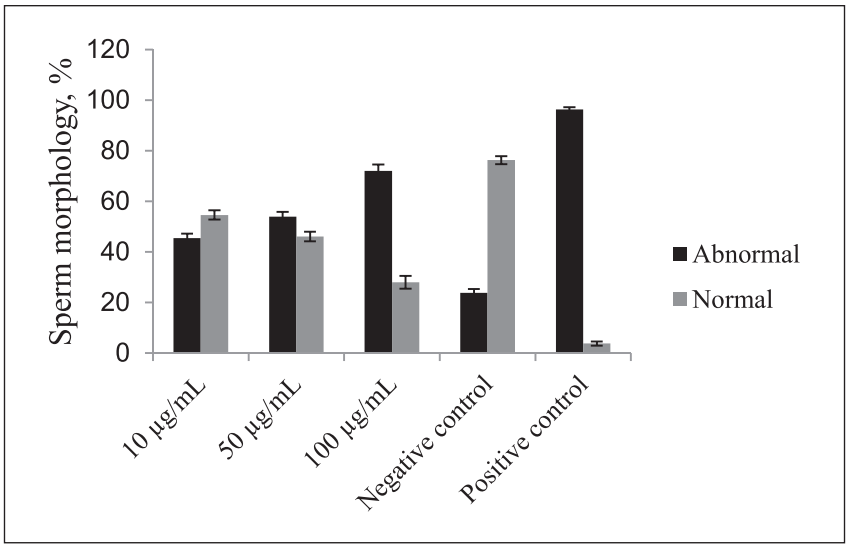

Fig. 2. Effect of biosynthesized nano-silver particles on sperm morphology. Total number of cells analysed per data entry, n=1500 spermatozoa
*abnormal sperm morphology included the numeration of either head, tail or multiple defects to the sperm morphology

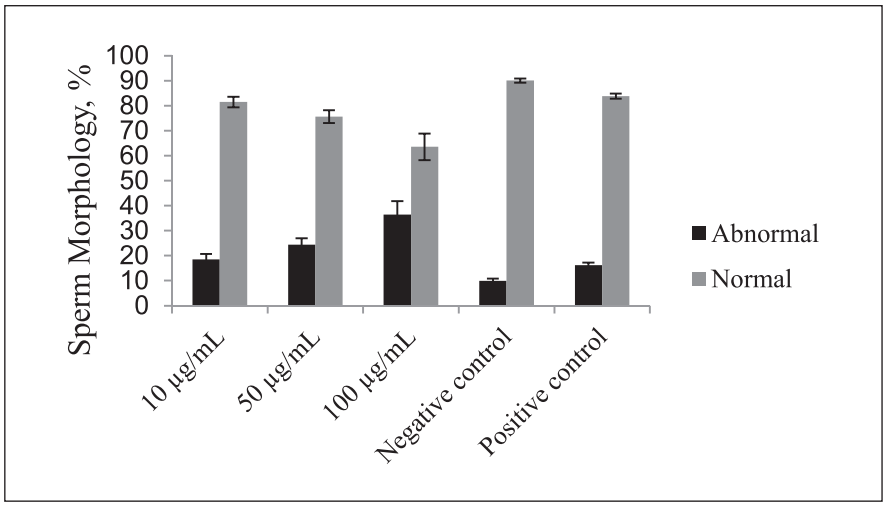

Fig. 3. Effect of biosynthesized nano-zinc oxide particles on sperm morphology. Total number of cells analysed per data entry, n=1500 spermatozoa
*abnormal sperm morphology included the numeration of either head, tail or multiple defects to the sperm morphology

2010). These findings are in accordance with a previous study (Braydich-Stolle *et al.*, 2010) that reported the negative effect of silver nanoparticles on the growth of male stem cells when they were exposed at concentrations greater than 10 µg/ml. The membrane solubilisation was observed as a dose dependent manner with percentages of non-viable sperm significantly increased for all treatment groups due to cytoarchitecture damage to the sperm membrane caused by the biosynthesized nanoparticles. It can be concluded that the reprotoxic effect increased and resulted in elevated occurrence of sperm with abnormal morphology due to membrane solubilisation as a direct result of increase in osmotic pressure of the incubation solution contributed by the presence of biosynthesized nanoparticles.

Sperm Motility

Observed results from the study show that as the concentration of biosynthesized nanoparticles increased, the percentage of non-motile sperm increased while the percentages of non-progressive sperm decreased. The opposing observations for non-motile and non-progressive sperm could be due to the contribution of non-progressive sperm

into the non-motile sperm count. Thus, the biosynthesized nanoparticles were able to negatively affect sperm motility. Previous studies have shown a positive relationship between the increasing concentration of nanoparticles used and the reduction of sperm motility values. Figure 4 shows the result of biosynthesized nano-silver particles and Figure 5 shows the result of biosynthesized nano-zinc oxide particles of differing concentrations with sperm motility. The reduction in sperm motility values could be due to a reduction of free thiol residues on the cell membrane after nanoparticle exposure as reported by (Taylor *et al.*, 2013). Thiol groups of cysteine residues in protein structures are important redox centres involved in multiple biological functions relating to sperm maturation (Dias *et al.*, 2014). The protein oxidation in the sperm maturation process is responsible for stabilization of sperm structure; protection against oxidative damage as well as induction of progressive sperm motility (Abarikwu *et al.*, 2010). Post hoc analysis showed that there is significant differences in sperm motility between each treatment of nano-silver particles at concentrations of 10 µg/ml, 50 µg/ml and 100 µg/ml ($p<0.05$, Fig. 4). The same trend was also observed for nano-zinc oxide particles where all treatment groups showed significant differences at concentrations of 10 µg/ml, 50 µg/ml and 100 µg/ml ($p<0.05$, Fig. 5). Highest non motile sperm count (80% non motile sperm) observed at 100 µg/mL nano-silver particles or 100 µg/mL nano-zinc oxide particles. It can be concluded that all treatment groups of nano-silver particles and biosynthesized nano-zinc oxide particles were able to not only affect the morphology but also the motility of sperm. The majority of the sperm within the sample were either rendered immotile or with non-directed movement that is mostly circular (non-progressive motility). Factors effecting motility could be due to pH, temperature, membrane integrity and osmolality of solution (Effer *et al.*, 2013). Based on the study, all treatments groups of biosynthesized nano-silver and nano-zinc oxide particles showed a decrease in the number of progressive sperm and an increase in percentage for non-motile sperm. Based on the post hoc analysis, it revealed that there is significant differences in sperm motility between, negative control group and all the treatment using biosynthesized nanoparticles treatment ($p<0.05$). This evidence shows that the biosynthesized nanoparticles affect the sperm motility, increasing sperm count with abnormal morphology although used at concentrations as low as 10 µg/ml (Ema *et al.*, 2010).

CONCLUSIONS

The exposure of nano-silver and nano-zinc oxide particles to sperm has proven to have deleterious effects on the sperm morphology, the viability of the sperm as well as its motility. An increase in nano-silver and nano-zinc oxide concentration induced greater reprotoxicity to sperm morphology, viability and sperm motility. The reprotoxic characteristic is important in order for silver nanoparticles to be considered as a new spermicidal agent. The observed effect on the sperm is largely attributed to the fact that the biosynthesized nanoparticles were able to disrupt the integrity of the sperm membrane, thus causing a decline in the selected sperm parameters. Sperm motility declined at nanoparticle mass dose of 10 µg/ml (corresponding to ~14000 nanoparticles per sperm cell) (Taylor *et al.*, 2013). Sperm fertilising ability (inferred from motility parameters)

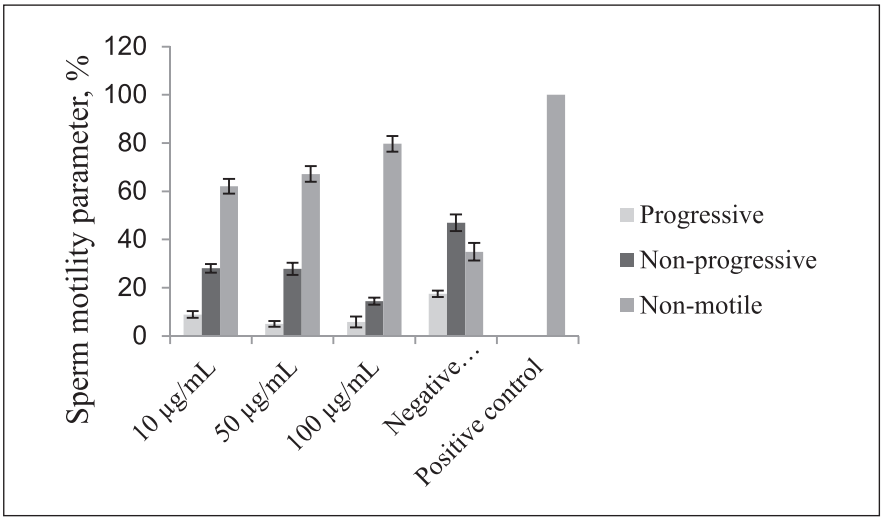

Fig. 4. Effect of biosynthesized nano-silver particles on sperm motility. Total number of cells analysed per data entry, n=1500 spermatozoa.

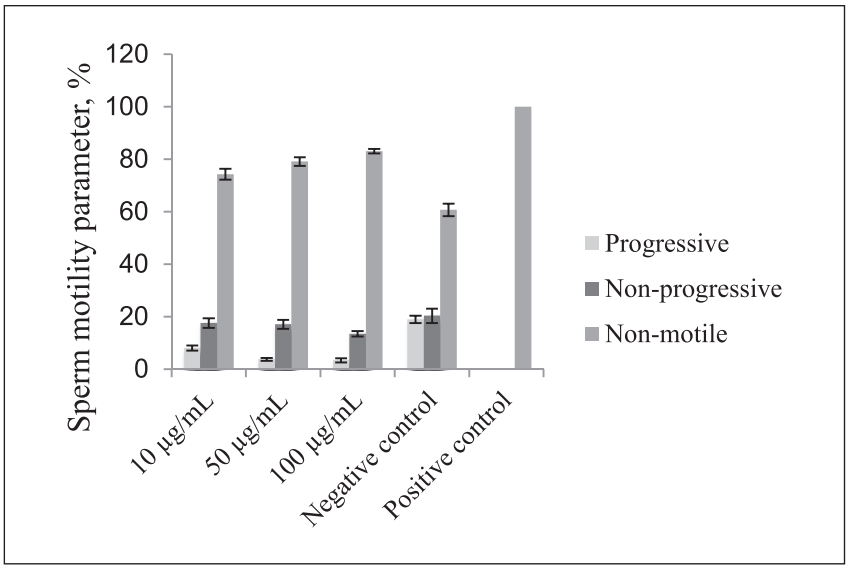

Fig. 5. Effect of biosynthesized nano-zinc oxide particles on sperm motility. Total number of cells analysed per data entry, n=1500 spermatozoa.

decreased after exposure to 10 µg/ml of nanoparticles, indicating that nanoparticles interfere with membrane properties necessary for fertilisation. In conclusion, nanoparticles may impair key sperm functions solely by interacting with the sperm surface membrane. The biosynthesized nanoparticles may have infiltrated the spermatozoa membrane, and caused the osmolarity of the cell to increase tremendously thus affected its integrity. This could be an interest for further study to elucidate the mechanism of action by which the nanoparticles are able to damage the sperm.

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